

FORM PTO-1390 (REV 12-29-99)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 41613
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO (If known, see 37 CFR 1.5) 09/831627	
INTERNATIONAL APPLICATION NO. PCT/US99/24017	INTERNATIONAL FILING DATE 12 November 1999	PRIORITY DATE CLAIMED 13 November 1998	
TITLE OF INVENTION A SENSITIVE SCREENING SYSTEM FOR ENVELOPE-DEFECTIVE RECOMBINANT VIRUS			
APPLICANT(S) FOR DO/EO/US Anatoly Bukovsky & Luigi Naldini			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
<ol style="list-style-type: none"> <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1). <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <ol style="list-style-type: none"> <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). <input type="checkbox"/> has been transmitted by the International Bureau. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <ol style="list-style-type: none"> <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). <input type="checkbox"/> have been transmitted by the International Bureau. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. <input type="checkbox"/> have not been made and will not be made. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). 			
Items 11. to 16. below concern document(s) or information included:			
<ol style="list-style-type: none"> <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. <input type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. <input type="checkbox"/> A substitute specification. <input type="checkbox"/> A change of power of attorney and/or address letter. <input checked="" type="checkbox"/> Other items or information: 3 sheets of formal drawings First Page of the Published Application Form PCT/IB/308 International Search Report Form PCT/IPEA/402 			

17. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$970.00
 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$840.00
 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$690.00
 International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$670.00
 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$96.00

CALCULATIONS PTO USE ONLY**ENTER APPROPRIATE BASIC FEE AMOUNT =**

\$ 690.00

Surcharge of **\$130.00** for furnishing the oath or declaration later than 20 30 months from the earliest claimed priority date (37 CFR 1.492(e)).

\$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	3 - 20 =	0	X \$18.00	\$
Independent claims	1 - 3 =	0	X \$78.00	\$
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00	\$

TOTAL OF ABOVE CALCULATIONS =

\$ 690.00

Reduction of 1/2 for filing by small entity, if applicable. Small Entity Status applies

\$ 345.00

SUBTOTAL =

\$ 345.00

Processing fee of **\$130.00** for furnishing the English translation later than 20 30 months from the earliest claimed priority date (37 CFR 1.492(f)).

\$

TOTAL NATIONAL FEE =

\$ 345.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property

\$

TOTAL FEES ENCLOSED =

\$ 345.00

Amount to be refunded:	\$
charged:	\$

a. A check in the amount of \$ 345.00 to cover the above fees is enclosed.

b. Please charge my Deposit Account No. 0 in the amount of \$ to cover the above fees
 A duplicate copy of this sheet is enclosed.

c. The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 18-2220. A duplicate copy of this sheet is enclosed.

The Declaration/Power of Attorney and Assignment will be filed shortly.

Priority is claimed from US 60/108,168 filed 13 November 1998.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

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REGISTRATION NUMBER

A Sensitive Screening System For Envelope-Defective Recombinant Virus

FIELD OF INVENTION

A sensitive screening system identifies envelope-defective recombinant viruses originating during production of lentiviral or retroviral vectors.

BACKGROUND OF THE INVENTION

Generally, recombinant viruses are replication-defective. However, such recombinant viruses still may be harmful to vector production in several ways. First, recombinant viruses may be propagated in vector producer cells. Second, recombinant viruses can interfere with the transduction of the vector by competing during encapsidation of the viral particles. Moreover, recombinant viruses may be harmful to a vector recipient due to the transfer of vector packaging functions. That may cause toxicity or an immune reaction in the transduced cells and host. There also may be an increase in the risk of additional recombination events which eventually could lead to generation of replication-competent retrovirus.

Such recombinant viruses may originate from the recombination of two or more of the constructs used to produce the vector, or from one such construct and endogenous retroviral sequences expressed in vector producer or target cells. Recombinants generally contain viral cis-acting sequences required for encapsidation and transfer to the target cells. Recombinants also generally contain the gag/pol gene sequences of a retrovirus or lentivirus.

The extent to which defective recombinants, such as envelope defective recombinants, contaminate batches of vector produced for clinical use or influence the performance of a vector producer system, is often unknown. The risk of defective versus replication competent recombinants occurring is

increased with the new split-genome packaging cell lines for retroviral vectors and with the use of vector pseudotyping due to the lack of any overlap between the constructs encoding the envelope and the gag/pol genes. Although that implies a lower risk of replication-competent recombinants occurring, the 5 recombination between the gag/pol construct and the transfer vector carrying the foreign gene of interest may still occur and generate envelope-defective recombinants that go unnoticed in conventional screening.

10 Defective recombinants contaminating vector lots used in clinical trials also may be responsible for false positive results in certain assays used to monitor replication-competent recombinants in the recipients.

15 Sensitive detection and early elimination of defective recombinants thus is crucial to validate and to maintain the performance of a vector producer system, as well as to prove the purity and safety of a vector batch. However, as defective recombinants are replication-defective as well, routine assays used to monitor retroviral recombinants that are based on amplification through replication in the indicator cell line(s) cannot detect the defective recombinants.

SUMMARY OF THE INVENTION

20 The invention describes an amplification method which detects replication-defective recombinants that is based on transcomplementation in an indicator cell line which provides the missing packaging functions, for example, an envelope gene to detect envelope-defective recombinants. An important feature of the complementing envelope is little if no interference with superinfection of the indicator cells thereby allowing under certain 25 circumstances amplification by pseudoreplication of the recombinant in a homogenous culture.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts how an envelope-defective recombinant can be generated from a packaging plasmid and a transfer vector carrying HIV sequences. The upper diagram depicts the wild-type HIV-1 genome. In the 5 diagrams, LTR is a long terminal repeat; SD is a splice donor site; GAG is the group antigen coding sequence; PRO is the protease coding sequence; POL is the polymerase coding sequence; VIF and NEF are accessory genes; CMV is the cytomegalovirus enhancer/promoter; poly A is a polyadenylation site; the delta ENV designation indicated deletion of the envelope coding sequence; SA is a splice acceptor site; prom is a promoter; Transgene is a foreign gene of interest; ψ is an encapsidation signal sequence; TAT and REV are regulatory genes; and RRE is Rev Responsive Element. The solid blocks indicate the HIV open reading frames or functional genes in the three reading frames.

Figure 2 depicts the results of assays aimed at determining the sensitivity of the method of interest.

Figure 3 depicts the results of viral amplification in the absence or presence of the VSV G envelope.

DETAILED DESCRIPTION OF THE INVENTION

20 The detection of replication defective retroviral recombinants, particularly of lentivirus-based vectors, rests with providing in trans a complementing function for that suspected of being defective in the recombinants. For example, if detection of envelope-defective recombinants is desired, a means for providing envelope protein is utilized. That can be 25 accomplished by developing transcomplementing cell lines which express one or more components needed for producing virus particles, such as an envelope protein.

An indicator cell line expressing a transcomplementing protein, such as, envelope protein, tat protein, rev protein or a combination thereof may be constructed and used as a screening agent in the instant invention.

Preferably the indicator cell lines are stable, transformed cells that express the one or more transcomplementing factors. Generally a stable, transformed cell in one wherein the transgene encoding the desirable factor is integrated into the host genome. That can be accomplished, for example, by transfection or by using a vector known to integrate into a host genome, such as, a retroviral vector, a transposon-based vector or an adeno-associated viral vector to carry the transgene.

Moreover, because a foreign gene product, such as an envelope protein, may be toxic to the host cell, it is desirable to regulate the expression of the transgene. For example, an inducible promoter may be used to control the expression of the transgene. Such inducible promoters are known in the art.

Otherwise, the making and maintenance of the transformed cell as well as the vectors of interest are as known in the art using materials readily available to the artisan. Any of a variety of host cells can be used. Moreover, the vectors and transgenes are known and the artisan can rely on known methods to construct a vector of interest.

Essentially, any known vector carrying a transcomplementing gene of interest and any suitable host cell can be used. Known inducible regulation systems can be used to regulate the expression of the transcomplementing gene. Also, any known method for detecting virus or expression of a gene product originating from the defective recombinant can be used, such as an immunoassay for a gag protein.

The 293G cell line which expresses the envelope protein G of vesicular stomatitis virus (VSV) (Ory et al., Proc. Natl. Acad. Sci. 93:11400-11406, 1996) can be used to detect envelope-defective viruses wherein the entry thereof

in target cells can be mediated by said protein G. The G protein is of interest because that envelope glycoprotein has been found to complement a wide range of viruses. Expression of the VSV G protein in 293G is controlled in a tetracycline regulated manner.

5 The VSV G cell line can be used, for example, to amplify minimal amounts of envelope-defective recombinant viruses that can nevertheless express and transfer the gag/pol genes of HIV. With that tetracycline regulated system, the cells are maintained in the presence of tetracycline which suppresses the expression of VSV G. Removal of tetracycline from the culture medium
10 maintaining 293G cells results in induction of VSV G protein expression on the surface of the indicator cells thereby allowing for efficient pseudotyping and/or entry of the released viral particles. The particles in turn are capable of superinfecting the indicator cells which leads to amplification of the input viral recombinant.

15 The VSV G envelope is particularly useful by endowing the viral particles with a very high infectivity thereby enhancing the sensitivity and robustness of the assay.

20 Amplification of virus not only by viruses carrying the envelope glycoprotein but also by viruses which lack or do not express an envelope glycoprotein has been observed. Thus, in the case of the VSV G protein, the expression of G protein at the surface of target cells is sufficient to mediate productive infection independent of expression within the viral membrane.

25 In view thereof, the instant invention relates to a method wherein screening of vector recipients following therapy can be accommodated. The method also can be used to amplify other enveloped viruses for which natural cellular receptors have yet to be identified.

 The instant assay will find use, for example, in the production of efficient and safe HIV-based lentiviral vectors. The growth of the recombinant particles

can be detected by immunoenzymatic assays detecting, for example, the HIV p24 gag antigen, or by RNA-PCR assays for detecting the HIV gag gene. Then, the presence of defective recombinants can be monitored by the use of the instant indicator cells.

5 The instant method also can be used to identify partial recombinant viruses that express and transfer the gag/pol genes of other retroviruses, for example, the lentivirus, such as, the various SIV's, FIV, HIV-2, visna-maedi virus, caprine arthritis-encephalitis virus, BIV and equine infectious anemia virus, and other retrovirus, such as, spumavirus, murine leukemia and sarcoma 10 viruses, other mammalian C-type viruses, such as FeLV and simian sarcoma virus, HERV's, B-type virus, such as mouse mammary tumor virus, D-type virus, HTLV's, bovine leukemia virus and avian leukosis-sarcoma viruses, such as Rous sarcoma virus and avian myeloblastosis virus. The detection system of the recombinant particle would be adjusted to the genes of the selected virus.

15 In another embodiment of the invention, the indicator cell line transcomplements one or both of the essential regulatory genes of lentivirus, tat and rev, in addition to the envelope gene. Such a system is useful to identify partial recombinants that contain the gag/pol genes of a lentivirus but do not express those genes efficiently, because for example, the regulatory genes 20 required for efficient expression of the gag/pol genes are lacking or are defective. Suitable assays would be those detecting expression of gag or pol.

25 The amplification provided by the instant method in the case of detecting envelope-defective recombinants arises from the significantly more efficient viral entry mediated by the complementing envelope proteins as compared to the homologous or parental gene product. Thus, another use of the instant method is a fast selection of viral gene variants of a desired phenotype, such as drug-resistance or growth advantage. The selection could be performed without

the need of actually producing an infectious viral construct as a complementing envelope protein can be produced by a cell, such as an indicator cell of interest.

The invention now will be exemplified in the following non-limiting examples.

5

EXAMPLES

For validation of the detection system, an envelope-defective recombinant was constructed by molecular cloning using known techniques. A VSV G protein expressing construct, pMD.G, which does not contain HIV sequences, was used (Naldini et al., Science 272:263-267, 1996a). Viral particles were generated by co-transfected an envelope-defective recombinant construct and the VSV.G expressing construct into 293T cells (Naldini et al., 1996a, supra; Proc. Natl. Acad. Sci. 93:11382-11388, 1996b). The recombinant construct was an HIV-based vector containing all but envelope sequences.

1000 100 10 1

20

Control virus was produced by means of transient transfection of the envelope-defective recombinant plasmid R8.7 delE and the VSV.G expressor plasmid pMD G (Naldini et al., 1996a, *supra*) into 293T cells. The R8.7 plasmid was constructed by cloning the BclI-XhoI fragment of plasmid pCMVDR8.74 (Dull et al., *J. Virol.* 72:8463-8471, 1998), which contains the HIV gag, pol, tat and rev genes but no accessory genes, into R8 (Gallay et al., *Cell* 83:569-576, 1995).

25

Cells were seeded in 10 cm dishes 24 hours before infection and washed 2 hours before transfection. Culture medium (IMDM, 10% FCS) was replaced at 14 hours and transfected-conditioned medium was collected at 36 hours post transfection. The conditioned medium was cleared by low-speed centrifugation (1500g) and passed through 0.45 µm filters. The amount of viral particles in the medium was measured by immunocapture assay for the HIV-1 p24 gag antigen (DuPont).

Titration of the viral particles on the VSV.G indicator cells by limiting dilution permitted an estimate of the sensitivity of the assay and provided a means to determine the amount of envelope-defective recombinants in vector preparations.

Indicator 293G cells (Ory et al., *supra*) and control 293-cells were seeded in 6-well plates at approximately 30% confluence 24 hours before infection. Immediately before infection, calls were washed with fresh medium. Control virus was diluted serially 10-fold in the medium without tetracycline and 1 ml of each dilution was added to each well. Cultured medium from infected cells were replaced regularly and amplification of recombinants was monitored by measuring p24 antigens in the supernatant. Infected cells were split 1/5 after confluence was reached.

As provided in Figure 2, the system is capable of detecting an inoculum of viral particles encapsidating an envelope-defective construct at a level of less than 20 fg p24 equivalent (lowest dilution used) in a 15 day incubation period. A gradual increase in p24 antigen concentration was observed in the culture supernatant on the indicated day after infection. No amplification of the recombinant was seen when 293 cells lacking the VSV envelope where infected with the same amount of viral particles.

The data of Figure 3 demonstrate the ability of the 293G cell to support virus amplification after initial infection by virus lacking envelope glycoprotein. Virions were produced by transient co-transfection of a plasmid encoding the HIV derivative containing deletion of the env gene and of either carrier DNA or the pMD.G plasmid encoding VSV G. Virions were normalized for p24 content, serially diluted (20, 0.5 and 0.125 ng/ml) and incubated with either induced 293/G cells or control 293 cells. As the expression of G in the 293G/ cell line is regulated by tetracycline, the cells were maintained in the absence of tetracycline 24 hours prior to infection.

During a two week period, supernatants of infected cells were monitored for p24 content as a measure of viral amplification. The data in Figure 3 are the results observed at the end of a two week period.

No p24 was detected in the supernatant of 293 cells incubated with
5 envelope-defective virions.

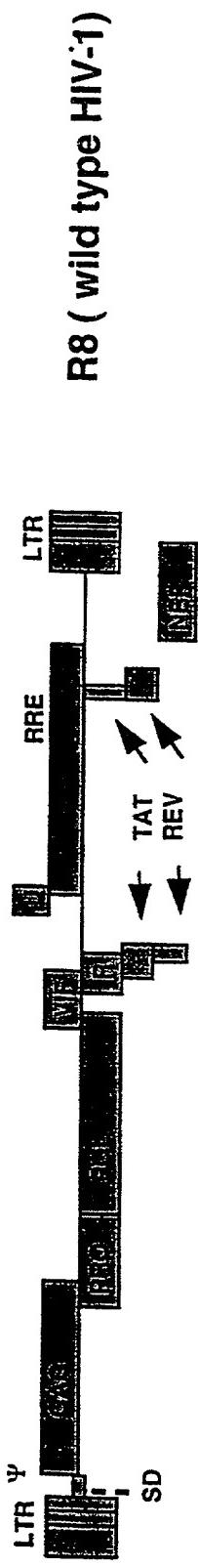
As expected, 293 cells infected with VSV G pseudotyped viruses produced low levels of p24 which was proportional to the input amount of virus and was not amplified throughout the incubation period. On the other hand, 293G cells generated increasing amounts of p24 with identical kinetics whether
10 pseudotyped or envelope defective virus was used for the initial infection.

We claim:

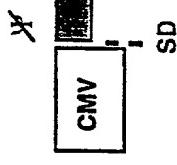
1. A method of amplifying an envelope-defective retrovirus by exposing said retrovirus to a cell comprising a virus envelope gene, wherein virus envelope encoded by said gene complements said retrovirus.
2. The method of claim 1, wherein said virus envelope is expressed at the surface of a virus particle produced by said cell.
3. The method of claim 1, wherein said virus envelope is expressed by said cell.

PCT/US99/24017

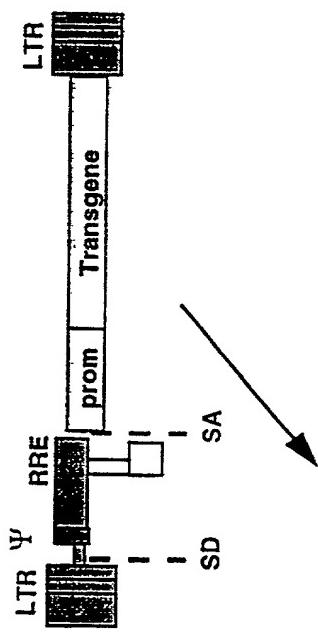
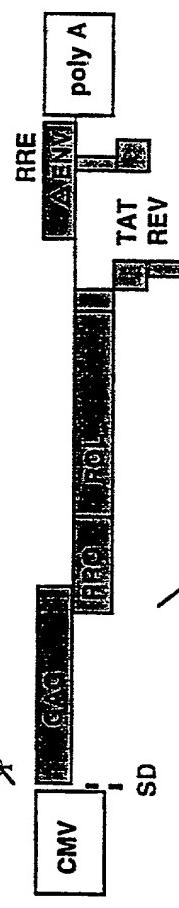
A



Vector Packaging Construct



Transfer Vector Construct



Envelope-defective recombinant



B

Figure 1

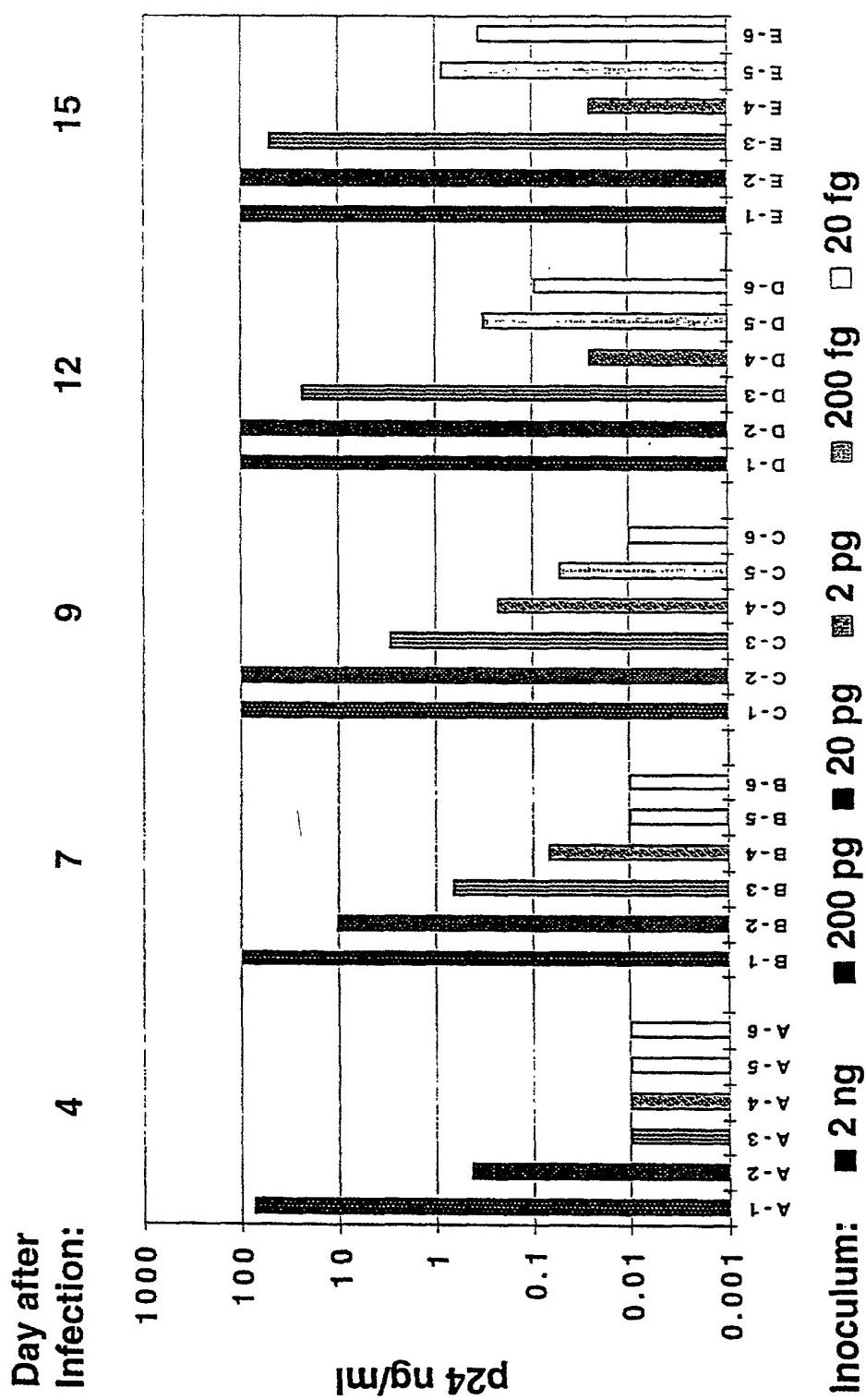


Figure 2. Amplification of partial recombinants lacking the Env gene.

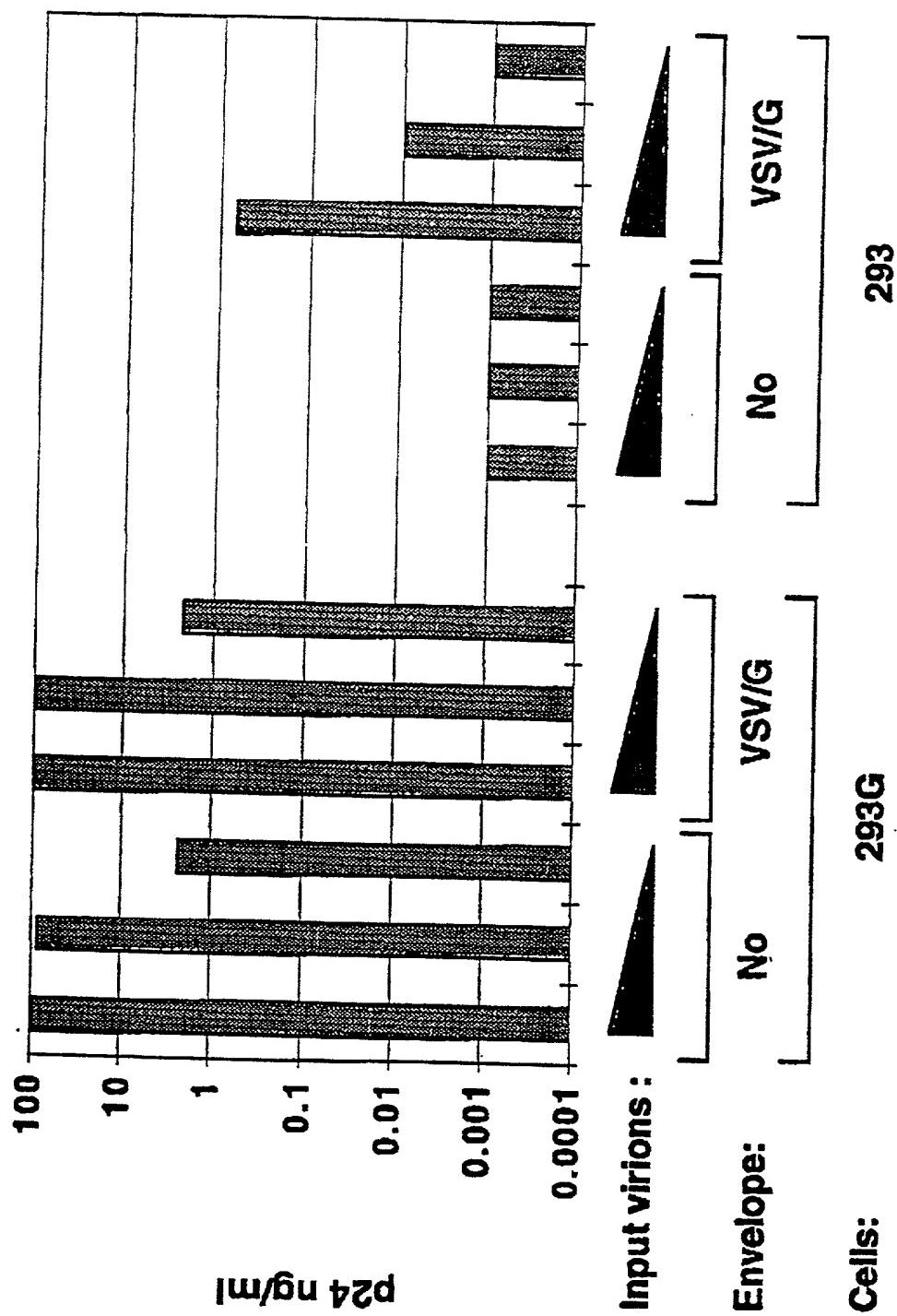


Figure 3 Efficient Amplification of virions lacking Env glycoprotein

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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

As the below named inventor(s), I/we declare that:

This declaration is directed to:

- The attached application, or
 Application No. 09/831,627 | _____, filed on 11 May 2001 | _____,
 as amended on _____ (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

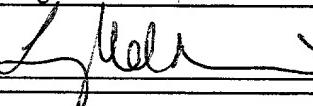
I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including material information which became available between the filing date of the prior application and the National or PCT International filing date of the continuation-in-part application, if applicable; and

All statements made herein of my/own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor one: Luigi Naldini |

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Inventor two: Anatoly Bukovsky |

Signature: | Citizen of: |

Inventor three: |

Signature: | Citizen of: |

Inventor four: |

Signature: | Citizen of: |

Additional inventors are being named on _____ additional form(s) attached hereto.

Burden Hour Statement: This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is used by the public to file (and the PTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This form is estimated to take 1 minute to complete. This time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

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- The attached application, or
 Application No. 09/831,627, filed on 11 May 2001,
 as amended on _____ (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including material information which became available between the filing date of the prior application and the National or PCT International filing date of the continuation-in-part application, if applicable; and

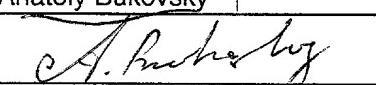
All statements made herein of my/own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

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Inventor two: Anatoly Bukovsky

Signature:  Citizen of: Russia

Inventor three: _____

Signature: _____ Citizen of: _____

Inventor four: _____

Signature: _____ Citizen of: _____

Additional inventors are being named on _____ additional form(s) attached hereto.

Burden Hour Statement: This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is used by the public to file (and the PTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This form is estimated to take 1 minute to complete. This time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

Initial Information Data Sheet

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Application Information

Title Line One::

A Sensitive Screening System for

Title Line Two::

Envelope-Defective Recombinant Virus

Total Drawing Sheets::

3

Formal Drawings?::

Yes

Application Type::

Utility

Docket Number::

41613

Representative Information

Registration Number::

33,981

11